Antioxidant Activity of Pea Bean (*Phaseolus vulgaris* L.) Extract

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Antioxidative activity of pea bean (*Phaseolus vulgaris* L.) extract was evaluated by using a linoleic acid system, and the methanol extract exhibited strong antioxidative activity as measured by the thiocyanate method. The crude methanol extract was partitioned between the *n*-butanol phase (BP) and the water phase (WP). Then, the antioxidative activity of the BP and the WP was determined by using a linoleic acid system. The WP showed strong antioxidative activity, while BP showed only weak activity as measured by the thiocyanate method. Next, the synergistic antioxidative action of WP with α-tocopherol was examined by using linoleic acid and liposome systems. The WP had a synergistic effect with α-tocopherol in both the food model and liposome systems. For purification and isolation of the antioxidative substances of the pea bean, preparative high-performance liquid chromatography was carried out with an octadecylsilyl column. Five fractions were collected, and antioxidative activity was determined in a linoleic acid system. Although fraction 1 had strong activity by the thiocyanate method, the purification of this active fraction was difficult; therefore, the partly characterized active fraction was investigated. The contents of total phenolics and sugars were 0.31 ± 0.01 mg/g of fraction 1 and 406.1 ± 0.1 mg/g, respectively. The ninhydrin chromogenic reaction was positive, and the ultraviolet absorption spectral λ max value in distilled water was 264.0 nm, indicating that the water-soluble antioxidative components from pea bean may be a new type of antioxidant. Isolation and identification are currently being investigated.

KEY WORDS: Antioxidant, *Phaseolus vulgaris* L., synergistic effect.

Lipid peroxidation is known as one of the major factors in the deterioration of foods during storage and processing. In addition, it is thought to induce physiological obstruction causing cell aging or carcinogenesis (1). The addition of antioxidants has become popular as a means of increasing the shelf life of food products and improving the stability of lipids and lipid-containing foods by preventing loss of sensory and nutritional quality.

Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been used widely in foods but are suspected to be carcinogenic (2,3). Tocopherols and ascorbic acid are now widely used as safe natural antioxidants. However, the antioxidant activities of tocopherols and ascorbic acid are lower than those of synthetic antioxidants. Hence, much research has been conducted to find safe antioxidants with high antioxidative activity from natural sources (4–9).

Pea bean (*Phaseolus vulgaris* L.) is cultivated throughout the world for its pods and seeds and consumed in various dishes. However, there are few reports about the antioxidative activity of pea bean extract. The effects of navy bean (*P. vulgaris* L.) hull extract on the oxidative stability of edible oil were reported by Onyeneho and Hettiarachchy (10), but this report did not include chemical studies of the bean antioxidants.

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In this paper, we describe our investigation of the antioxidative activity of pea bean extract and its synergistic antioxidative action with α-tocopherol, together with chemical studies involving purification of the antioxidants and partial characterization of the active fraction.

MATERIALS AND METHODS

Materials. Pea bean (*P. vulgaris* L.) with a white seed coat, also named "Ohtebo" in Japan for use in white bean paste, was used in all experiments. The seed was purchased from Hirukawa Co. Ltd. (Nagoya, Japan). Egg lecithin and linoleic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). α-Tocopherol was obtained from Eisai Co. Ltd. (Tokyo, Japan). BHA and phytic acid were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Daidzin and genistin prepared from soybean were obtained from Sugiyama Jogakuen University (Nagoya, Japan).

Extraction and fractionation of crude antioxidants. The ground bean sample (500 g) was defatted with 5 L of *n*-hexane. The residue was dried and extracted with 5 L of methanol twice and then filtered. The filtrate was concentrated *in vacuo*. The crude sample was suspended in 250 mL of water. The suspension was extracted with 500 mL of *n*-butanol and partitioned between *n*-butanol phase (BP) and water phase (WP). BP and WP were concentrated, lyophilized and used for antioxidative assays.

Antioxidative activity in a linoleic acid system. Each antioxidant sample (200 μg) was added to a solution mixture of linoleic acid (0.13 mL), 99.0% distilled ethanol (10 mL) and 0.2 M phosphate buffer (pH 7.0, 10 mL) and the total volume was adjusted to 25 mL with distilled water. The solution was mixed in a conical flask and incubated at 37°C. At intervals during incubation, the degree of oxidation was measured in duplicate by the thiocyanate method (11) by reading the absorbance at 500 nm after coloring with $FeCl_2$ and thiocyanate.

Synergistic antioxidative action in the linoleic acid system. α-Tocopherol (40 μg) was added to each mixture just described, then an amount of WP (15 μg, 30 μg or 40 μg) was added to each flask. The antioxidative activity was measured in duplicate by using the thiocyanate (11) and the thiobarbituric acid (TBA) methods (12).

Synergistic antioxidative action in a liposome system. Egg lecithin (100 mg) was sonicated in an ultrasonic cleaner (Bransonic 220; Branson Ultrasonics Co. Ltd., Danbury, CT) with 10 mM phosphate buffer (pH 7.4). The resulting multilamellar vesicles (MLV) were sonicated in a cuphorn-type sonicator (Insonator 201M; Kubota Works, Tokyo, Japan) at 120 W for 15 min, by which process small unilamellar vesicles (SUV) were obtained. The SUV solution (10 mg liposome/mL), $CuSO_4$, phosphate buffer (pH 7.4) and antioxidants were mixed to produce a final concentration of 1 mg liposome/mL, 1 μM $CuSO_4$ and 1 mM phosphate buffer (pH 7.4). α-Tocopherol was added to the reaction mixture, then an amount of WP (2 μg, 10 μg, 50 μg or 100 μg) was added to the reaction mixture. The antioxidative activity was measured

in duplicate by the method of Buege and Aust (13) after incubation for 4 h at 37°C. The results were expressed in nmoles of malondialdehyde (MDA) equivalent (eq.) per mg lipid and were calculated by using an extinction coefficient of $1.56 \times 10^5\ M^{-1}cm^{-1}$.

High-performance liquid chromatography (HPLC). Preparative HPLC was carried out on a JASCO Twincle HPLC (Japan Spectroscopic Co, Ltd., Tokyo, Japan) by using a Develosil ODS-10 (20 mm × 250 mm) column (Nomura Chemical Co, Ltd., Seto, Japan) with an ultraviolet (UV) spectrophotometric detector (JASCO UVIDEC-100; Japan Spectroscopic) set at 264 nm. The WP was eluted first with an isocratic condition of 0.1% trifluoroacetic acid (TFA) in water for 30 min and then with a linear gradient ranging from 0.1% TFA in water to 0.1% TFA in 7:3 methanol/water over 60 min. The flow rate was 5.0 mL/min. The HPLC-separated fractions were assayed in duplicate for antioxidative activity by the thiocyanate method.

Partial characterization of the active fraction. Quantitation of total phenolics and sugar contents was done according to the method described by the Association of Official Analytical Chemists (14) and by the phenol-sulfuric acid method (15), respectively. The ninhydrin chromogenic reaction was carried out by the method of Moore and Stein (16). The UV absorption was carried out on a Spectrophotometer, Model UV270 (Shimazu Works, Kyoto, Japan).

Heat treatment of active fraction. The active fraction (10 mg) was dissolved in 2 mL of 0.2 M phosphate buffer (pH 7.0) in screw-cap tubes. Tests were run in duplicate, then heated in boiling water for 1 h. After the treatment, the tubes were cooled, 40 μL of the treated solution (0.2 mg of WP) was added to the linoleic acid system, and antioxidative activity was measured by the thiocyanate method. The extent of the activity was compared with unheated WP (0.2 mg).

Statistics. Statistical analysis was performed by using Student's *t*-test (17).

RESULTS AND DISCUSSION

Antioxidative activity of crude extracts, n-BP and WP. Antioxidative activities of the methanol extracts of pea bean in the linoleic acid system as measured by the thiocyanate method are shown in Figure 1. The experiment was replicated four times, and reported values are the mean ± SD. A control containing no added antioxidants or additives represents 100% lipid peroxidation. As shown, pea bean methanol extract exhibited strong antioxidative activity. The activity of methanol extracts (0.2 mg or 1.0 mg) was stronger than 0.2 mg α-tocopherol ($P < 0.01$ and $P < 0.001$, respectively).

The antioxidative activities in the linoleic acid system of BP and WP are shown in Figure 2. The results of the antioxidative assay are averages of duplicate measurements. The experiment was repeated in triplicate, and typical results are shown from one of the three independent experiments. WP showed strong activity, but BP did not show appreciable activity. These data suggest that the water-soluble antioxidants must be present in the pea bean methanol extract.

Synergistic antioxidative action of WP with α-tocopherol. One of the major factors in the loss of viability in stored seeds has been ascribed to damage in the intracellular membrane structures induced by lipid peroxidation. In general, seeds contain a great variety of natural antioxidants (tocopherols, carotenoids and many other phenolic compounds) that are though to inhibit lipid peroxidation and protect against damage to membrane functions (18).

The tocopherol content of the pea bean was relatively low (3.4 mg/100 g seeds), indicating that the pea bean may

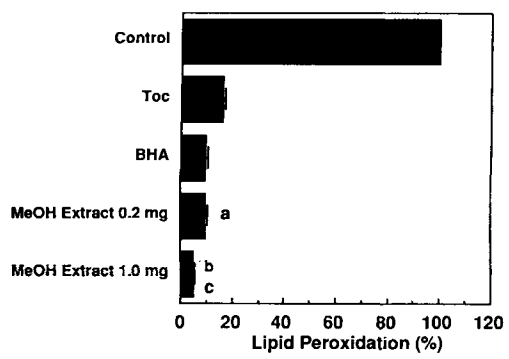

FIG. 1. Antioxidative activity of methanol extracts of pea bean (*Phaseolus vulgaris* L.), butylated hydroxyanisole (BHA) and α-tocopherol (Toc) (200 μg), as measured by the thiocyanate method. [a]$P < 0.01$, [b]$P < 0.001$, compared with Toc. [c]$P < 0.01$, compared with BHA.

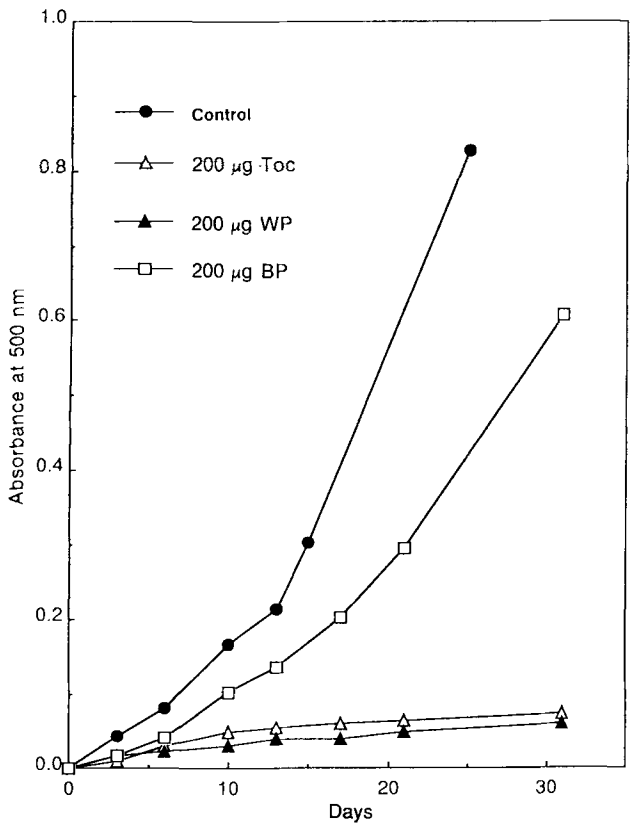

FIG. 2. Antioxidative activity of the water phase (WP) of pea bean methanolic extract and the *n*-butanol phase (BP), as measured by the thiocyanate method. Toc, tocopherol.

have antioxidative defense systems with different endogenous antioxidants, such as phenolics or endogenous synergists with tocopherols. Therefore, the synergistic effects of WP on the antioxidative activity of α-tocopherol were studied in linoleic acid and liposome systems.

Figure 3 shows the synergistic effects of the WP and α-tocopherol on the antioxidative activity in the linoleic acid system by using the thiocyanate method. The results of the antioxidative assay are averages of duplicate measurements. The experiment was repeated in triplicate, and typical results are shown from one of the three independent experiments. The addition of both WP and α-tocopherol (40 μg, each) per 25-mL flask resulted in stronger antioxidative activity than either tocopherol singly at 40 or 200 μg. Figure 4 shows the same tendency by the TBA method.

To get more information on the formation and behavior of lipid peroxides in biological systems, the liposome system was used for evaluation of antioxidative activity. The synergistic effects of WP with α-tocopherol in the liposome system are shown in Figure 5. The experiment was repeated four times, and reported values are the mean ± SD. The addition of 50 or 500 μg of WP to α-tocopherol (2 μg) resulted in stronger antioxidative activity than that of 2 μg of α-tocopherol alone ($P < 0.001$) when lipid peroxidation was induced by 1 μM $CuSO_4$. These results show that the WP has a synergistic antioxidative action with α-tocopherol, both in the linoleic acid and in the liposome systems, and may play an important role in protecting against damage to membrane functions within the plant. Furthermore, the synergistic effect may be used for increasing the shelf life of foods.

HPLC. Preparative HPLC was carried out for purification and isolation of the antioxidants. A chromatogram of the WP is shown in Figure 6, as is the division of the extract into fractions 1, 2, 3, 4 and 5.

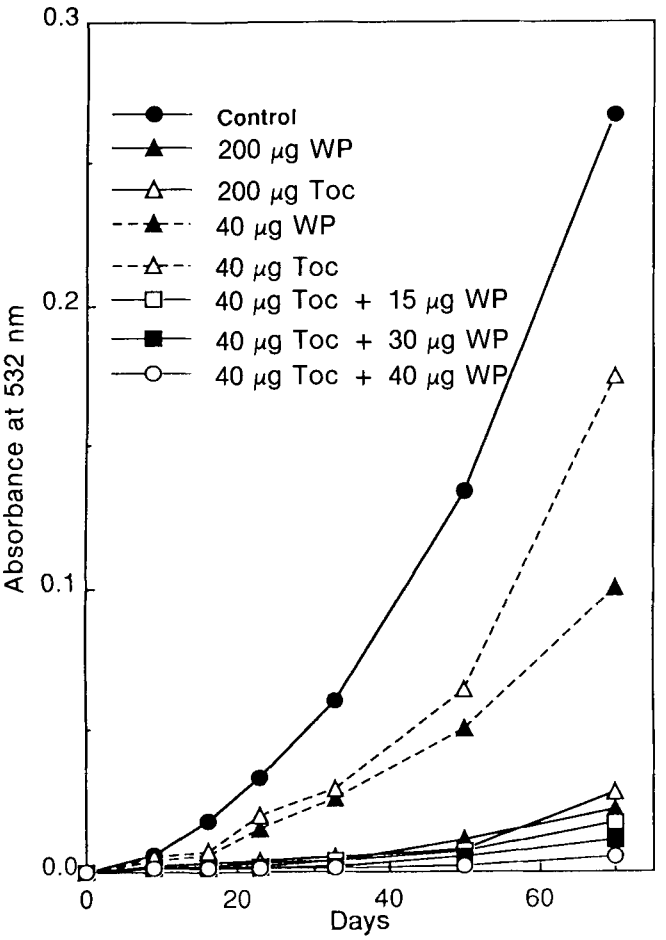

FIG. 4. Synergistic antioxidative action of the water phase (WP) of pea bean methanolic extract with α-tocopherol (Toc) in the linoleic acid system, as measured by the thiobarbituric acid method.

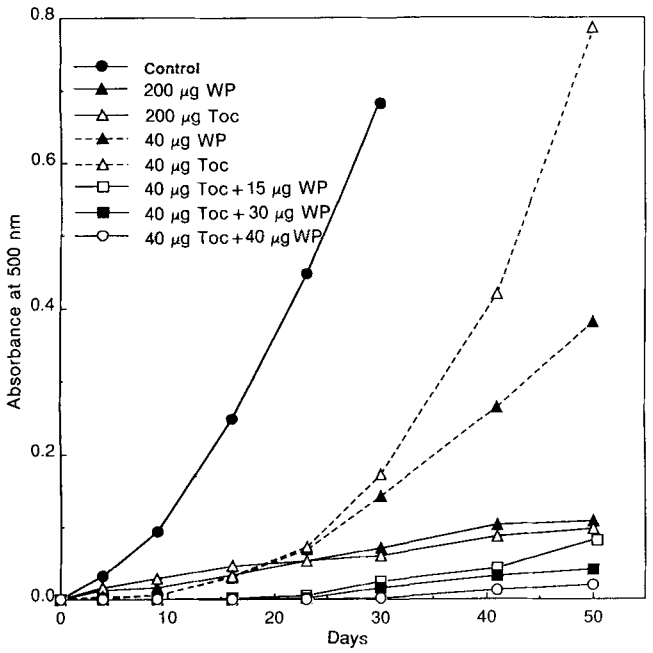

FIG. 3. Synergistic antioxidative action of the water phase (WP) of pea bean methanolic extract with α-tocopherol (Toc) in the linoleic acid system, as measured by the thiocyanate method.

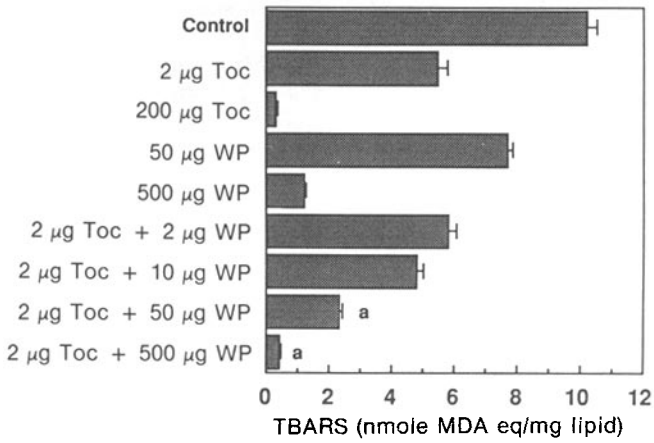

FIG. 5. Synergistic antioxidative action of the water phase (WP) of pea bean methanolic extract with α-tocopherol (Toc) in the liposome system, as measured by the method of Buege and Aust (13). Lipid peroxidation was induced by 1 μM $CuSO_4$. [a]$P < 0.001$, compared with 2 μg Toc. TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde.

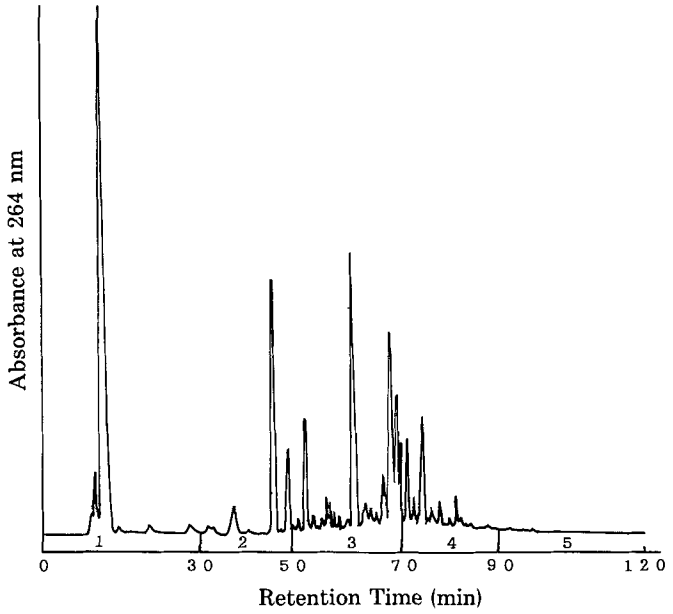

FIG. 6. High-performance liquid chromatography chromatogram of the water phase of pea bean methanolic extract. Conditions: column, Develosil ODS-10 (20 mm × 250 mm); eluent, 0.1% trifluoroacetic acid (TFA) in water, linear gradient 0.1% TFA in water to 0.1% in 7:3 methanol/water over 60 min; flow rate, 5.0 mL/min; detector ultraviolet 264 nm. Fractions 1, 2, 3, 4 and 5 are indicated.

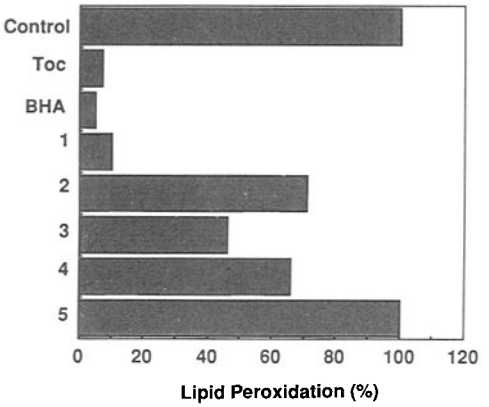

FIG. 7. Antioxidative activity measured by the thiocyanate method of a control, α-tocopherol (Toc), butylated hydroxyanisole (BHA) and of five antioxidant fractions separated by high-performance liquid chromatography (200 μg each) of the water phase.

The antioxidative activities of each fraction (200 μg) and of controls containing either no antioxidants, α-tocopherol or BHA were determined by the thiocyanate method (Fig. 7). The control was considered to have 100% lipid peroxidation, with other values being reported as a percentage peroxidation in relation to the control. The results are the averages of duplicate measurements. Fraction 1 possessed strong activity, but fractions 2, 3, 4 and 5 showed only weak activity. Fraction 1 was eluted with isocratic conditions of 0.1% TFA in water. Because this fraction is extremely soluble in water and not adsorbed onto the ODS column, further separation of the antioxidants in fraction 1 on the ODS column was difficult. A method of isolation and identification of the components in this fraction is being developed.

Partial characterization of the active fraction. Because purification of fraction 1 on the reverse-phase column was difficult, the active fraction has been characterized only partly. Although the UV absorption spectral λ max value was 264.0 nm, the total phenolic amount of fraction 1 was low (0.03%), as shown in Table 1. Standards of daidzin and genistin, isoflavone glycosides with antioxidant activity found in soybean (*Glycine max* L.) (19), were compared to the compounds in fraction 1 by HPLC with a reverse-phase Develosil ODS column, but they did not match. The results suggest that the antioxidants in fraction 1 may not be phenolic substances.

The total sugar content in fraction 1 was 406.1 ± 0.1 mg/g (Table 1). Given the high sugar content, fraction 1 may contain phytic acid (inositol hexaphosphoric acid), which has previously been identified in pea bean (20). It has been reported that phytic acid acts as an antioxidant by chelating metal ions (21,22). But phytic acid was not detected in fraction 1 by comparing the retention time of phytic acid with the HPLC unknown peaks (data not shown).

TABLE 1

Total Phenolics, Sugar Content, Ninhydrin Chromogenic Reaction and Ultraviolet (UV) Spectral Data (λ max) of Fraction 1 of the Water Phase of Pea Bean Methanolic Extract

Total phenolics[a] (mg/g of fraction 1)	0.31 ± 0.01
Total sugar[a] (mg/g of fraction 1)	406.1 ± 0.1
Ninhydrin reaction	Positive
UV spectral data, λ max (nm)	264.0

[a]Reported values are mean ± SD (n = 3).

Because the ninhydrin reaction was positive, fraction 1 must contain amino acids or peptides, although the details of identification are not clear.

Antioxidative activity in fraction 1, as measured by the thiocyanate method, was completely stable, even after heating for 1 h at 100°C (results not shown). Given these results, we assume that the water-soluble antioxidative components of pea bean must be a new type of antioxidant, although their structures are still unknown. Because of their superior stability, the antioxidative components contained in the pea beans might be used to increase the shelf life of processed bean products as well as to protect against oxidative damage in living systems (23). Isolation and identification are now under investigation. We hope to report the structure of the antioxidants in the near future.

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